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(54) Title: ANTISENSE MODULATION OF p38 MITOGEN ACTIVATED PROTEIN KINASE EXPRESSION

(57) Abstract

Compositions and methods for the treatment and diagnosis of diseases or conditions amenable to treatment through modulation of expression of a gene encoding a p38 mitogen–activated protein kinase (p38 MAPK) are provided. Methods for the treatment and diagnosis of diseases or conditions associated with aberrant expression of one or more p38 MAPKs are also provided.

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ANTISENSE MODULATION OF p38 MITOGEN ACTIVATED PROTEIN KINASE EXPRESSION

FIELD OF THE INVENTION

This invention relates to compositions and methods for modulating expression of p38 mitogen activated protein kinase genes, a family of naturally present cellular genes involved in signal transduction, and inflammatory and apoptotic responses. This invention is also directed to methods for inhibiting inflammation or apoptosis; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to treatment of diseases or conditions associated with expression of p38 mitogen activated protein kinase genes.

BACKGROUND OF THE INVENTION

Cellular responses to external factors, such as growth factors, cytokines, and stress conditions, result in altered gene expression. These signals are transmitted from the cell surface to the nucleus by signal transduction pathways. Beginning with an external factor binding to an appropriate receptor, a cascade of signal transduction events is initiated. These responses are mediated through activation of various enzymes and the subsequent activation of specific transcription factors. These activated transcription factors then modulate the expression of specific genes.

The phosphorylation of enzymes plays a key role in the transduction of extracellular signals into the cell. Mitogen activated protein kinases (MAPKs), enzymes which effect such phosphorylations are targets for the action of growth factors, hormones, and other agents involved in cellular metabolism, proliferation and differentiation (Cobb *et al.*, *J. Biol. Chem.*, **1995**, *270*, 14843). Mitogen activated protein kinases were initially discovered due to

their ability to be tyrosine phosphorylated in response to exposure to bacterial lipopolysaccharides or hyperosmotic conditons (Han *et al*, *Science*, **1994**, *265*, 808). These conditions activate inflammatory and apoptotic responses mediated by MAPK. In general, MAP kinases are involved in a variety of signal transduction pathways (sometimes overlapping and sometimes parallel) that function to convey extracellular stimuli to protooncogene products to modulate cellular proliferation and/or differentiation (Seger *et al.*, *FASEB J.*, **1995**, *9*, 726; Cano *et al.*, *Trends Biochem. Sci.*, **1995**, *20*, 117).

One of the MAPK signal transduction pathways involves the MAP kinases p38α and p38β(also known as CSaids Binding Proteins, CSBP). These MAP kinases are responsible for the phosphorylation of ATF-2, MEFC2 and a variety of other cellular effectors that may serve as substrates for p38 MAPK proteins (Kummer *et al*, *J. Biol. Chem.*, **1997**, *272*, 20490). Phosphorylation of p38 MAPKs potentiates the ability of these factors to activate transcription (Raingeaud *et al*, *Mol. Cell Bio.*, **1996**, *16*, 1247; Han *et al*, *Nature*, **1997**, *386*, 296). Among the genes activated by the p38 MAPK signaling pathway is IL-6 (De Cesaris, P., *et al.*, *J. Biol. Chem.*, **1998**, *273*, 7566-7571).

Besides p38α and p38β, other p38 MAPK family members have been described, including p38γ (Li *et al*, *Biochem. Biophys. Res. Commun.*, **1996**, *228*, 334), and p38δ (Jiang *et al*, *J. Biol. Chem.*, **1997**, *272*, 30122). The term "p38" as used herein shall mean a member of the p38 MAPK family, including but not limited to p38α, p38β, p38γ and p38δ, their isoforms (Kumar *et al*, *Biochem. Biophys. Res. Commun.*, **1997**, *235*, 533) and other members of the p38 MAPK family of proteins whether they function as p38 MAP kinases *per se* or not.

Modulation of the expression of one or more p38 MAPKs is desirable in order to interfere with inflammatory or apoptotic responses associated with disease states and to modulate the transcription of genes stimulated by ATF-2, MEFC2 and other p38 MAPK phosphorylation substrates.

Inhibitors of p38 MAPKs have been shown to have efficacy in animal models of arthritis (Badger, A.M., *et al.*, *J. Pharmacol. Exp. Ther.*, **1996**, *279*, 1453-1461) and angiogenesis (Jackson, J.R., *et al.*, *J. Pharmacol. Exp. Ther.*, **1998**, *284*, 687-692). MacKay, K. and Mochy-Rosen, D. (*J. Biol. Chem.*, **1999**, *274*, 6272-6279) demonstrate that an inhibitor of p38 MAPKs prevents apoptosis during ischemia in cardiac myocytes, suggesting that p38 MAPK inhibitors can be used for treating ischemic heart disease. p38 MAPK also is required for T-cell HIV-1 replication (Cohen *et al*, *Mol. Med.*, **1997**, *3*, 339) and may be a useful target for AIDS therapy. Other diseases believed to be amenable to treatment by inhibitors of p38 MAPKs are disclosed in US Patent No. 5,559,137, herein incorporated by reference.

Therapeutic agents designed to target p38 MAPKs include small molecule inhibitors and antisense oligonucleotides. Small molecule inhibitors based on pyridinyl imidazole are described in US Patent No. 5,670,527; 5,658,903; 5,656,644; 5,559,137; 5,593,992; and 5,593,991. WO 98/27098 and WO 99/00357 describe additional small molecule inhibitors, one of which has entered clinical trials. Other small molecule inhibitors are also known.

Antisense therapy represents a potentially more specific therapy for targeting p38 MAPKs and, in particular, specific p38 MAPK isoforms. Nagata, Y., *et al.* (*Blood*, **1998**, *6*, 1859-1869) disclose an antisense phosphothioester oligonucleotide targeted to the translational start site of mouse p38b (p38β). Aoshiba,

K., *et al.* (*J. Immunol.*, **1999**, *162*, 1692-1700) and Cohen, P.S., *et al.* (*Mol. Med.*, **1997**, *3*, 339-346) disclose a phosphorothioate antisense oligonucleotide targeted to the coding regions of human p38α, human p38β and rat p38.

There remains a long-felt need for improved compositions and methods for modulating the expression of p38 MAP kinases.

SUMMARY OF THE INVENTION

The present invention provides antisense compounds which are targeted to nucleic acids encoding a p38 MAPK and are capable of modulating p38 MAPK expression. The present invention also provides oligonucleotides targeted to nucleic acids encoding a p38 MAPK. The present invention also comprises methods of modulating the expression of a p38 MAPK, in cells and tissues, using the oligonucleotides of the invention. Methods of inhibiting p38 MAPK expression are provided; these methods are believed to be useful both therapeutically and diagnostically. These methods are also useful as tools, for example, for detecting and determining the role of p38 MAPKs in various cell functions and physiological processes and conditions and for diagnosing conditions associated with expression of p38 MAPKs.

The present invention also comprises methods for diagnosing and treating inflammatory diseases, particularly rheumatoid arthritis. These methods are believed to be useful, for example, in diagnosing p38 MAPK-associated disease progression. These methods employ the oligonucleotides of the invention. These methods are believed to be useful both therapeutically, including prophylactically, and as clinical research and diagnostic tools.

DETAILED DESCRIPTION OF THE INVENTION

p38 MAPKs play an important role in signal

transduction in response to cytokines, growth factors and other cellular stimuli. Specific responses elicited by p38 include inflammatory and apoptotic responses. Modulation of p38 may be useful in the treatment of inflammatory diseases, such as rheumatoid arthritis.

The present invention employs antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding a p38 MAPK, ultimately modulating the amount of a p38 MAPK produced. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding a p38 MAPK.

The antisense compounds may be used to modulate the function of a particular p38 MAPK isoform, e.g. for research purposes to determine the role of a particular isoform in a normal or disease process, or to treat a disease or condition that may be associated with a particular isoform. It may also be desirable to target multiple p38 MAPK isoforms. In each case, antisense compounds can be designed by taking advantage of sequence homology between the various isoforms. If an antisense compound to a particular isoform is desired, then the antisense compound is designed to a unique region in the desired isoform's gene sequence. With such a compound, it is desirable that this compound does not inhibit the expression of other isoforms. Less desirable, but acceptable, are compounds that do not "substantially" inhibit other isoforms. By "substantially", it is intended that these compounds do not inhibit the expression of other isoforms greater than 25%; more preferred are compounds that do not inhibit other isoforms greater than 10%. If an antisense compound is desired to target multiple p38 isoforms, then regions of significant homology between the isoforms can be used.

This relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding a p38 MAPK; in other words, a p38 MAPK gene or RNA expressed from a p38 MAPK gene. p38 MAPK mRNA is presently the preferred target. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is

typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding p38, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. This region is a preferred target region. The open

reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene) and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene). mRNA splice sites may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions may also be preferred targets.

Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment and, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA.

The overall effect of interference with mRNA function is modulation of p38 MAPK expression. In the context of this invention "modulation" means either inhibition or stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression as taught in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an immunoprecipitation assay of protein

expression, as taught in the examples of the instant application. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding a p38 MAPK, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Furthermore, since the oligonucleotides of this invention hybridize specifically to nucleic acids encoding particular isoforms of p38 MAPK, such assays can be devised for screening of cells and tissues for particular p38 MAPK isoforms. Such assays can be utilized for diagnosis of diseases associated with various p38 MAPK isoforms. Provision of means for detecting hybridization of oligonucleotide with a p38 MAPK gene or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of p38 MAPK may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples from patients suspected of having an inflammatory disease such as rheumatoid arthritis. The ability of the oligonucleotides of the present invention to inhibit inflammation may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell

suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal. Similarly, the present invention can be used to distinguish p38 MAPK-associated diseases, from diseases having other etiologies, in order that an efficacious treatment regime can be designed.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). Preferred embodiments comprise at least an 8-nucleobase portion of a sequence of an antisense compound which inhibits the expression of a p38 mitogen activated kinase. As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes

of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of some preferred modified oligonucleotides envisioned for this invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioates (usually abbreviated in the art as P=S) and those with CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2 [known as a methylene(methylimino) or MMI backbone], CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 and O-N(CH_3)-CH_2-CH_2 backbones, wherein the native phosphodiester (usually abbreviated in the art as P=O) backbone is represented as O-P-O-CH_2). Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent No. 5,034,506). Further preferred are oligonucleotides with NR-C(*)-CH_2-CH_2, CH_2-NR-C(*)-CH_2, CH_2-CH_2-NR-C(*), C(*)-NR-CH_2-CH_2 and CH_2-C(*)-NR-CH_2 backbones, wherein "*" represents O or S (known as amide backbones; DeMesmaeker *et al.*, WO 92/20823, published

November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen et al., *Science*, *254*, 1497 (1991); U.S. Patent No. 5,539,082). Other preferred modified oligonucleotides may contain one or more substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, OCH_3OCH_3, $OCH_3O(CH_2)_nCH_3$, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-O-methoxyethyl [which can be written as 2'-O-$CH_2CH_2OCH_3$, and is also known in the art as 2'-O-(2-methoxyethyl) or 2'-methoxyethoxy] [Martin et al., *Helv. Chim. Acta*, *78*, 486 (1995)]. Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-propoxy (2'-$OCH_2CH_2CH_3$), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of the 5' terminal nucleotide.

Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

The oligonucleotides of the invention may additionally or alternatively include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine and 5-methylcytosine, as well as synthetic nucleobases, e.g., 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6(6-aminohexyl)adenine and 2,6-diaminopurine [Kornberg, A., DNA Replication, 1974, W.H. Freeman & Co., San Francisco, 1974, pp. 75-77; Gebeyehu, G., et al., *Nucleic Acids Res., 15*, 4513 (1987)]. 5-methylcytosine (5-me-C) is presently a preferred nucleobase, particularly in combination with 2'-O-methoxyethyl modifications.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N^6 position of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., *et al., Nucleic Acids Res.*, 1987, *15*, 4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety [Letsinger et al., *Proc. Natl. Acad. Sci. USA,*, *86*, 6553 (1989)], cholic acid [Manoharan et al., *Bioorg. Med. Chem. Let.*, *4*, 1053

(1994)], a thioether, e.g., hexyl-*S*-tritylthiol [Manoharan et al., *Ann. N.Y. Acad. Sci.*, *660*, 306 (1992); Manoharan et al., *Bioorg. Med. Chem. Let.*, *3*, 2765 (1993)], a thiocholesterol [Oberhauser et al., *Nucl. Acids Res.*, *20*, 533 (1992)], an aliphatic chain, e.g., dodecandiol or undecyl residues [Saison-Behmoaras et al., *EMBO J.*, *10*, 111 (1991); Kabanov et al., *FEBS Lett.*, *259*, 327 (1990); Svinarchuk et al., *Biochimie.*, *75*, 49(1993)], a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate [Manoharan et al., *Tetrahedron Lett.*, *36*, 3651 (1995); Shea et al., *Nucl. Acids Res.*, *18*, 3777 (1990)], a polyamine or a polyethylene glycol chain [Manoharan et al., *Nucleosides & Nucleotides*, *14*, 969 (1995)], or adamantane acetic acid [Manoharan et al., *Tetrahedron Lett.*, *36*, 3651 (1995)], a palmityl moiety [Mishra et al., *Biochim. Biophys. Acta*, *1264*, 229 (1995)], or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety [Crooke et al., *J. Pharmacol. Exp. Ther.*, *277*, 923 (1996)]. Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, as disclosed in U.S. Patent No. 5,138,045, No. 5,218,105 and No. 5,459,255, the contents of which are hereby incorporated by reference.

The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An

additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNAse H-mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted). Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention

contain a 2'-O-methoxyethyl (2'-O-$CH_2CH_2OCH_3$) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide. According to the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl (-O-$CH_2CH_2OCH_3$) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred. Through use of such modifications, active oligonucleotides have been identified which are shorter than conventional "first generation" oligonucleotides active against p38. Oligonucleotides in accordance with this invention are from 5 to 50 nucleotides in length. In the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having from 5 to 50 monomers.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-

methoxyethyl oligonucleotides [Martin, P., *Helv. Chim. Acta, 78*, 486 (1995)]. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids.

Pharmaceutically acceptable "salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto [see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, *66*:1 (1977)].

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic

acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories,

i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, *8*:91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7*:1). One or more penetration enhancers from one or more of these broad categories may be included.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded

by one or more outer layers made up of lipids arranged in a bilayer configuration [see, generally, Chonn et al., *Current Op. Biotech.*, *6*, 698 (1995)].

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the

invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents, particularly those used for tumor and cancer treatment. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine,taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., pp. 1206-1228, Berkow *et al.*, eds., Rahay, N.J., 1987). When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with

the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect on the treated mammal. This amount, which will be apparent to the skilled artisan, will depend upon the type of mammal, the age and weight of the mammal, the type of disease to be treated, perhaps even the gender of the mammal, and other factors which are routinely taken into consideration when treating a mammal with a disease. A therapeutic effect is assessed in the mammal by measuring the effect of the compound on the disease state in the animal. For example, if the disease to be treated is cancer, therapeutic effects are assessed by measuring the

rate of growth or the size of the tumor, or by measuring the production of compounds such as cytokines, production of which is an indication of the progress or regression of the tumor.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1: Synthesis of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β-cyanoethyldiisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^{3}H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-methoxy oligonucleotides are synthesized using 2'-methoxy β-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Other 2'-alkoxy oligonucleotides were synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides are synthesized as described in Kawasaki et al., *J. Med. Chem.*, *36*, 831 (1993). Briefly, the protected nucleoside N^6-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-ß-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-α-fluoro atom is introduced by a S_N2-

displacement of a 2'-ß-O-trifyl group. Thus N^6-benzoyl-9-ß-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N^6-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-ß-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-anhydro-1-ß-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N^4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P., *Helv. Chim. Acta*, *78*,486 (1995). For ease of synthesis, the last nucleotide was a deoxynucleotide. 2'-O-$CH_2CH_2OCH_3$-cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

2,2'-Anhydro[1-(β-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 hours) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and

evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue

was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

<u>N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:</u>

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

<u>N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:</u>

N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure

fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods [Sanghvi et al., *Nucl. Acids Res., 21*, 3197 (1993)] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

<u>5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine</u>

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) are dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) is added in one portion. The reaction is stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicates a complete reaction. The solution is concentrated under reduced pressure to a thick oil. This is partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer is dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil is dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution is cooled to -10°C. The resulting crystalline product is collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of

white solid. TLC and NMR are used to check consistency with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor is added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) is added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) are added with manual stirring. The reactor is sealed and heated in an oil bath until an internal temperature of 160 °C is reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel is cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicates % conversion to the product. In order to avoid additional side product formation, the reaction is stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue is purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions are combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. TLC and NMR are used to determine consistency with pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with

triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819, 86%).

<u>5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine</u>

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) is dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) is added dropwise at -10°C to 0°C. After 1 hr the mixture is filtered, the filtrate is washed with ice cold CH_2Cl_2 and the combined organic phase is washed with water, brine and dried over anhydrous Na_2SO_4. The solution is concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which is then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1eg.) is added and the mixture for 1 hr. Solvent is removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam.

<u>5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine</u>

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-

formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) is dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) is added to this solution at 10°C under inert atmosphere. The reaction mixture is stirred for 10 minutes at 10°C. After that the reaction vessel is removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) is added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase is dried over anhydrous Na_2SO_4, evaporated to dryness. Residue is dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) is added and the reaction mixture is stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) is added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture is removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution is added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer is dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained is purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g).

<u>2'-O-(dimethylaminooxyethyl)-5-methyluridine</u>

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) is dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF is then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction is monitored by TLC (5% MeOH in CH_2Cl_2). Solvent is removed

under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) is dried over P_2O_5 under high vacuum overnight at 40°C. It is then co-evaporated with anhydrous pyridine (20mL). The residue obtained is dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) is added to the mixture and the reaction mixture is stirred at room temperature until all of the starting material disappeared. Pyridine is removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) is co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) is added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture is dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) is added. The reaction mixture is stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent is evaporated, then the residue is dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer is dried over anhydrous Na_2SO_4 and concentrated. Residue obtained is chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-

cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl) guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Patent No. 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al., *Acc. Chem. Res., 28,* 366 (1995). The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent No. 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al., *Science, 254,* 1497 (1991).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem., 266,* 18162 (1991). Results

obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 2: Human p38α Oligonucleotide Sequences

Antisense oligonucleotides were designed to target human p38α. Target sequence data are from the p38 MAPK cDNA sequence; Genbank accession number L35253, provided herein as SEQ ID NO: 1. Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of eight 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by six-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All 2'-MOE cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 1.

The human Jurkat T-cell line (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 growth media supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). HUVEC cells (Clonetics, San Diego, CA) were cultivated in endothelial basal media supplemented with 10% FBS (Hyclone, Logan, UT).

Jurkat cells were grown to approximately 75% confluency and resuspended in culture media at a density of 1×10^7 cells/ml. A total of 3.6×10^6 cells were employed for each treatment by combining 360 µl of cell suspension with oligonucleotide at the indicated concentrations to reach a final volume of 400 µl. Cells were then transferred to an electroporation cuvette and electroporated using an Electrocell Manipulator 600 instrument (Biotechnologies and Experimental Research, Inc.) employing 150 V, 1000 µF, at 13 Ω. Electroporated cells were then transferred to conical tubes containing 5

ml of culture media, mixed by inversion, and plated onto 10 cm culture dishes.

HUVEC cells were allowed to reach 75% confluency prior to use. The cells were washed twice with warm (37°C) OPTI-MEM™ (Life Technologies). The cells were incubated in the presence of the appropriate culture medium, without the growth factors added, and the oligonucleotide formulated in LIPOFECTIN® (Life Technologies), a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water. HUVEC cells were treated with 100 nM oligonucleotide in 10 μg/ml LIPOFECTIN®. Treatment was for four hours.

Total mRNA was isolated using the RNEASY® Mini Kit (Qiagen, Valencia, CA; similar kits from other manufacturers may also be used), separated on a 1% agarose gel, transferred to HYBOND™-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ), a positively charged nylon membrane, and probed. p38 MAPK probes were made using the Prime-A-Gene® kit (Promega Corporation, Madison, WI), a random primer labeling kit, using mouse p38α or p38β cDNA as a template. A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech (Palo Alto, CA), Catalog Number 9805-1. The fragments were purified from low-melting temperature agarose, as described in Maniatis, T., *et al.*, *Molecular Cloning: A Laboratory Manual*, **1989**. The G3PDH probe was labeled with REDIVUE™ ^{32}P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) and Strip-EZ labelling kit (Ambion, Austin, TX). mRNA was quantitated by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

TABLE 1:

Nucleotide Sequences of Human p38α Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
16486	AAGACCGGGCCCGGAATTCC	3	0001-0020	5'-UTR
16487	GTGGAGGCCAGTCCCCGGGA	4	0044-0063	5'-UTR
16488	TGGCAGCAAAGTGCTGCTGG	5	0087-0106	5'-UTR
16489	CAGAGAGCCTCCTGGGAGGG	6	0136-0155	5'-UTR
16490	TGTGCCGAATCTCGGCCTCT	7	0160-0179	5'-UTR
16491	GGTCTCGGGCGACCTCTCCT	8	0201-0220	5'-UTR
16492	CAGCCGCGGGACCAGCGGCG	9	0250-0269	5'-UTR
16493	CATTTTCCAGCGGCAGCCGC	10	0278-0297	AUG
16494	TCCTGAGACATTTTCCAGCG	11	0286-0305	AUG
16495	CTGCCGGTAGAACGTGGGCC	12	0308-0327	coding
16496	GTAAGCTTCTGACATTTCAC	13	0643-0662	coding
16497	TTTAGGTCCCTGTGAATTAT	14	0798-0817	coding
16498	ATGTTCTTCCAGTCAACAGC	15	0939-0958	coding
16499	TAAGGAGGTCCCTGCTTTCA	16	1189-1208	coding
16500	AACCAGGTGCTCAGGACTCC	17	1368-1387	stop
16501	GAAGTGGGATCAACAGAACA	18	1390-1409	3'-UTR
16502	TGAAAAGGCCTTCCCCTCAC	19	1413-1432	3'-UTR
16503	AGGCACTTGAATAATATTTG	20	1444-1463	3'-UTR
16504	CTTCCACCATGGAGGAAATC	21	1475-1494	3'-UTR
16505	ACACATGCACACACACTAAC	22	1520-1539	3'-UTR

[1] Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-) including "C" residues, 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. L35253, locus name

"HUMMAPKNS", SEQ ID NO. 1.

For an initial screen of human p38α antisense oligonucleotides, Jurkat cells were electroporated with 10 μM oligonucleotide. mRNA was measured by Northern blot. Results are shown in Table 2. Oligonucleotides 16496 (SEQ ID NO. 13), 16500 (SEQ ID NO. 17) and 16503 (SEQ ID NO. 20) gave 35% or greater inhibition of p38α mRNA.

TABLE 2

Inhibition of Human p38α mRNA expression in Jurkat Cells by Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
control	---	---	100%	0%
16486	3	5'-UTR	212%	---
16487	4	5'-UTR	171%	---
16488	5	5'-UTR	157%	---
16489	6	5'-UTR	149%	---
16492	7	5'-UTR	152%	---
16491	8	5'-UTR	148%	---
16492	9	5'-UTR	125%	---
16493	10	AUG	101%	---
16494	11	AUG	72%	28%
16495	12	coding	72%	28%
16496	13	coding	61%	39%
16497	14	coding	104%	---
16498	15	coding	88%	12%
16499	16	coding	74%	26%
16500	17	stop	63%	37%
16501	18	3'-UTR	77%	23%
16502	19	3'-UTR	79%	21%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
16503	20	3'-UTR	65%	35%
16504	21	3'-UTR	72%	28%
16505	22	3'-UTR	93%	7%

The most active human p38α oligonucleotides were chosen for dose response studies. Oligonucleotide 16490 (SEQ ID NO. 7) which showed no inhibition in the initial screen was included as a negative control. Jurkat cells were grown and treated as described above except the concentration of oligonucleotide was varied as indicated in Table 3. Results are shown in Table 3. Each of the active oligonucleotides showed a dose response effect with IC_{50}s around 10 nM. Maximum inhibition was approximately 70% with 16500 (SEQ ID NO. 17). The most active oligonucleotides were also tested for their ability to inhibit p38β. None of these oligonucleotides significantly reduced p38β mRNA expression.

TABLE 3

Dose Response of p38α mRNA in Jurkat cells to human p38α Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
control	---	---	---	100%	0%
16496	13	coding	2.5 nM	94%	6%
"	"	"	5 nM	74%	26%
"	"	"	10 nM	47%	53%
"	"	"	20 nM	41%	59%
16500	17	stop	2.5 nM	82%	18%

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
"	"	"	5 nM	71%	29%
"	"	"	10 nM	49%	51%
"	"	"	20 nM	31%	69%
16503	20	3'-UTR	2.5 nM	74%	26%
"	"	"	5 nM	61%	39%
"	"	"	10 nM	53%	47%
"	"	"	20 nM	41%	59%
16490	7	5'-UTR	2.5 nM	112%	---
"	"	"	5 nM	109%	---
"	"	"	10 nM	104%	---
"	"	"	20 nM	97%	3%

Example 3: Human p38β Oligonucleotide Sequences

Antisense oligonucleotides were designed to target human p38β. Target sequence data are from the p38β MAPK cDNA sequence; Genbank accession number U53442, provided herein as SEQ ID NO: 23. Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All 2'-MOE cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 4.

TABLE 4:

Nucleotide Sequences of Human p38β Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
17891	**CGACA**TGTCCGGAGC**AGAAT**	25	0006-0025	AUG
17892	**TTCAG**CTCCTGCCGG**TAGAA**	26	0041-0060	coding
17893	**TGCGG**CACCTCCCAC**ACGGT**	27	0065-0084	coding
17894	**CCGAA**CAGACGGAGC**CGTAT**	28	0121-0140	coding
17895	**GTGCT**TCAGGTGCTT**GAGCA**	29	0240-0259	coding
17896	**GCGTG**AAGACGTCCA**GAAGC**	30	0274-0293	coding
17897	**ACTTG**ACGATGTTGT**TCAGG**	31	0355-0374	coding
17898	**AACGT**GCTCGTCAAG**TGCCA**	32	0405-0424	coding
17899	**ATCCT**GAGCTCACAG**TCCTC**	33	0521-0540	coding
17900	**ACTGT**TTGGTTGTAA**TGCAT**	34	0635-0654	coding
17901	**ATGAT**GCGCTTCAGC**TGGTC**	35	0731-0750	coding
17902	**GCCAG**TGCCTCAGCT**GCACT**	35	0935-0954	coding
17903	**AACGC**TCTCATCATA**TGGCT**	37	1005-1024	coding
17904	**CAGCA**CCTCACTGCT**CAATC**	38	1126-1145	stop
17905	**TCTGT**GACCATAGGA**GTGTG**	39	1228-1247	3'-UTR
17906	**ACACA**TGTTTGTGCA**TGCAT**	40	1294-1313	3'-UTR
17907	**CCTAC**ACATGGCAAG**CACAT**	41	1318-1337	3'-UTR
17908	**TCCAG**GCTGAGCAGC**TCTAA**	42	1581-1600	3'-UTR
17909	**AGTGC**ACGCTCATCC**ACACG**	43	1753-1772	3'-UTR
17910	**CTTGC**CAGATATGGC**TGCTG**	44	1836-1855	3'-UTR

[1] Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-) including "C" residues, 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. U53442, locus name

"HSU53442", SEQ ID NO. 23.

For an initial screen of human p38β antisense oligonucleotides, HUVEC cells were cultured and treated as described in Example 2. mRNA was measured by Northern blot as described in Example 2. Results are shown in Table 5. Every oligonucleotide tested gave at least 50% inhibition. Oligonucleotides 17892 (SEQ ID NO. 26), 17893 (SEQ ID NO. 27), 17894 (SEQ ID NO. 28), 17899 (SEQ ID NO. 33), 17901 (SEQ ID NO. 35), 17903 (SEQ ID NO. 37), 17904 (SEQ ID NO. 38), 17905 (SEQ ID NO. 39), 17907 (SEQ ID NO. 41), 17908 (SEQ ID NO. 42), and 17909 (SEQ ID NO. 43) gave greater than approximately 85% inhibition and are preferred.

TABLE 5

Inhibition of Human p38β mRNA expression in Huvec Cells by Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
control	---	---	100%	0%
17891	25	AUG	22%	78%
17892	26	coding	10%	90%
17893	27	coding	4%	96%
17893	28	coding	13%	87%
17895	29	coding	25%	75%
17896	30	coding	24%	76%
17897	31	coding	25%	75%
17893	32	coding	49%	51%
17899	33	coding	5%	95%
17900	34	coding	49%	60%
17901	35	coding	15%	85%
17902	36	coding	49%	51%
17903	37	coding	11%	89%
17904	38	stop	9%	91%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
17905	39	3'-UTR	14%	86%
17906	40	3'-UTR	22%	78%
17907	41	3'-UTR	8%	92%
17908	42	3'-UTR	17%	83%
17909	43	3'-UTR	13%	87%
17910	44	3'-UTR	26%	74%

Oligonucleotides 17893 (SEQ ID NO. 27), 17899 (SEQ ID NO. 33), 17904 (SEQ ID NO. 38), and 17907 (SEQ ID NO. 41) were chosen for dose response studies. HUVEC cells were cultured and treated as described in Example 2 except that the oligonucleotide concentration was varied as shown in Table 6. The Lipofectin®/Oligo ratio was maintained at 3µg Lipofectin®/100nM oligo, per ml. mRNA was measured by Northern blot as described in Example 2.

Results are shown in Table 6. Each oligonucleotide tested had an IC_{50} of less than 10 nM. The effect of these oligonucleotides on human p38α was also determined. Only oligonucleotide 17893 (SEQ ID NO. 27) showed an effect on p38α mRNA expression. The IC_{50} of this oligonucleotide was approximately 4 fold higher for p38α compared to p38β.

TABLE 6

Dose Response of p38β in Huvec cells to human p38β Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
control	---	---	---	100%	0%
17893	27	coding	10 nM	37%	63%

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
"	"	"	25 nM	18%	82%
"	"	"	50 nM	16%	81%
"	"	"	100 nM	19%	81%
17899	33	coding	10 nM	37%	63%
"	"	"	25 nM	23%	77%
"	"	"	50 nM	18%	82%
"	"	"	100 nM	21%	79%
17904	38	stop	10 nM	31%	69%
"	"	"	25 nM	21%	79%
"	"	"	50 nM	17%	83%
"	"	"	100 nM	19%	81%
17907	41	3'-UTR	10 nM	37%	63%
"	"	"	25 nM	22%	78%
"	"	"	50 nM	18%	72%
"	"	"	100 nM	18%	72%

Example 4: Rat p38α Oligonucleotide Sequences

Antisense oligonucleotides were designed to target rat p38α. Target sequence data are from the p38 MAPK cDNA sequence; Genbank accession number U73142, provided herein as SEQ ID NO: 45. Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages in the wings are phosphodiester (P=O). Internucleoside linkages in the central gap are phosphorothioate (P=S). All 2'-MOE cytosines and 2'-OH cytosines were 5-methyl-cytosines.

These oligonucleotide sequences are shown in Table 7.

bEND.3, a mouse endothelial cell line (gift of Dr. Werner Risau; see Montesano *et al.*, *Cell*, 1990, *62*, 435, and Stepkowski *et al.*, *J. Immunol.*, 1994, *153*, 5336) were grown in high-glucose DMEM (Life Technologies, Gaithersburg, MD) medium containing 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycinin. Cells were plated at approximately 2 x 10^5 cells per 100 mm dish. Within 48 hours of plating, the cells were washed with phosphate-buffered saline (Life Technologies). Then, Opti-MEM® medium containing 3 µg/mL LIPOFECTIN® and an appropriate amount of oligonucleotide were added to the cells. As a control, cells were treated with LIPOFECTIN® without oligonucleotide under the same conditions and for the same times as the oligonucleotide-treated samples.

After 4 hours at 37°C, the medium was replaced with high glucose DMEM medium containing 10% FBS and 1% Penicillin/Streptomycinin. The cells were typically allowed to recover overnight (about 18 to 24 hours) before RNA and/or protein assays were performed as described in Example 2. The p38α, p38β and G3PDH probes used were identical to those described in Example 2.

TABLE 7:

Nucleotide Sequences of Rat p38α Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
21844	CoToGoCoGsAsCsAsTsTsTsTsCsCsAsGoCoGoGoC	47	0001-0020	AUG
21845	GoGoToAoAsGsCsTsTsCsTsGsAsCsAsCoToToCoA	48	0361-0380	coding
21846	GoGoCoCoAsGsAsGsAsCsTsGsAsAsTsGoToAoGoT	49	0781-0800	coding
21871	CoAoToCoAsTsCsAsGsGsGsTsCsGsTsGoGoToAoC	50	0941-0960	coding
21872	GoGoCoAoCsAsAsAsGsCsTsAsAsTsGsAoCoToToC	51	1041-1060	coding
21873	AoGoGoToGsCsTsCsAsGsGsAsCsTsCsCoAoToToT	52	1081-1100	stop
21874	GoGoAoToGsGsAsCsAsGsAsAsCsAsGsAoAoGoCoA	53	1101-1120	3'-UTR
21875	GoAoGoCoAsGsGsCsAsGsAsCsTsGsCsCoAoAoGoG	54	1321-1340	3'-UTR
21876	AoGoGoCoTsAsGsAsGsCsCsCsAsGsGsAoGoCoCoA	55	1561-1580	3'-UTR
21877	GoAoGoCoCsTsGsTsGsCsCsTsGsGsCsAoCoToGoG	56	1861-1880	3'-UTR
21878	ToGoCoAoCsCsAsCsAsAsGsCsAsCsCsToGoGoAoG	57	2081-2100	3'-UTR
21879	GoGoCoToAsCsCsAsTsGsAsGsTsGsAsGoAoAoGoA	58	2221-2240	3'-UTR
21880	GoToCoCoCsTsGsCsAsCsTsGsAsTsAsGoAoGoAoA	59	2701-2720	3'-UTR
21881	ToCoToToCsCsAsAsTsGsGsAsGsAsAsAoCoToGoG	60	3001-3020	3'-UTR

[1] Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-); 2'-MOE cytosines and 2'-deoxy cytosine residues are 5-methyl-cytosines; "s" linkages are phosphorothioate linkages; "o" linkages are phosphodiester linkages.

[2] Co-ordinates from Genbank Accession No. U73142, locus name "RNU73142", SEQ ID NO. 45.

Rat p38α antisense oligonucleotides were screened in bEND.3 cells for inhibition of p38α and p38β mRNA expression. The concentration of oligonucleotide used was 100 nM. Results are shown in Table 8. Oligonucleotides 21844 (SEQ ID NO. 47), 21845 (SEQ ID NO. 48), 21872 (SEQ ID NO. 51), 21873 (SEQ ID NO. 52), 21875 (SEQ ID NO. 54), and 21876 (SEQ ID NO. 55) showed greater than approximately 70% inhibition of p38α mRNA with minimal effects on p38β mRNA levels. Oligonucleotide 21871 (SEQ ID NO. 50) inhibited both p38α and p38β levels greater than 70%.

TABLE 8

Inhibition of Mouse p38 mRNA expression in bEND.3 Cells by Chimeric (deoxy gapped) Mixed Backbone p38α Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% p38α mRNA INHIBITION	% p38β mRNA INHIBITION
control	---	---	0%	0%
21844	47	AUG	81%	20%
21845	48	coding	75%	25%
21871	50	coding	90%	71%
21872	51	coding	87%	23%
21873	52	stop	90%	3%
21874	53	3'-UTR	38%	21%
21875	54	3'-UTR	77%	---
21876	55	3'-UTR	69%	---
21877	56	3'-UTR	55%	13%
21878	57	3'-UTR	25%	10%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% p38α mRNA INHIBITION	% p38β mRNA INHIBITION
21879	58	3'-UTR	---	---
21881	60	3'-UTR	---	---

Several of the most active oligonucleotides were selected for dose response studies. bEND.3 cells were cultured and treated as described above, except that the concentration of oligonucleotide was varied as noted in Table 9.

Results are shown in Table 9.

TABLE 9

Dose Response of bEND.3 cells to rat p38β Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% p38α mRNA Inhibition	% p38β mRNA Inhibition
control	---	---	---	100%	0%
21844	47	AUG	1 nM	---	---
"	"	"	5 nM	---	---
"	"	"	25 nM	36%	8%
"	"	"	100 nM	80%	5%
21871	50	coding	1 nM	1%	---
"	"	"	5 nM	23%	4%
"	"	"	25 nM	34%	24%
"	"	"	100 nM	89%	56%
21872	51	stop	1 nM	---	---
"	"	"	5 nM	---	---
"	"	"	25 nM	35%	---
"	"	"	100 nM	76%	1%
21873	52	stop	1 nM	---	53%

"	"	"	5 nM	---	31%
"	"	"	25 nM	54%	28%
"	"	"	100 nM	92%	25%
21875	54	3'-UTR	1 nM	---	11%
"	"	"	5 nM	---	16%
"	"	"	25 nM	33%	2%
"	"	"	100 nM	72%	4%

Example 5: Mouse p38β Oligonucleotide Sequences

Antisense oligonucleotides were designed to target mouse p38β. Target sequence data are from a mouse EST sequence; Genbank accession number AI119044, provided herein as SEQ ID NO: 61. Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages in the wings are phosphodiester (P=O). Internucleoside linkages in the central gap are phosphorothioate (P=S). All 2'-MOE cytosines and 2'-OH cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 10.

TABLE 10:

Nucleotide Sequences of Mouse p38β

Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]
100800	CoAoCoAoGsAsAsGsCsAsGsCsTsGsGsAoGoCoGoA	63	0051-0070
100801	ToGoCoGoGsCsAsCsCsTsCsCsCsAsTsAoCoToGoT	64	0119-0138
100802	CoCoCoToGsCsAsGsCsCsGsCsTsGsCsGoGoCoAoC	65	0131-0150
100803	GoCoAoGoAsCsTsGsAsGsCsCsGsTsAsGoGoCoGoC	66	0171-0190
100804	ToToAoCoAsGsCsCsAsCsCsTsTsCsTsGoGoCoGoC	67	0211-0230
100805	GoToAoToGsTsCsCsTsCsCsTsCsGsCsGoToGoGoA	68	0261-0280
100806	AoToGoGoAsTsGsTsGsGsCsCsGsGsCsGoToGoAoA	69	0341-0360
100807	GoAoAoToTsGsAsAsCsAsTsGsCsTsCsAoToCoGoC	70	0441-0460
100808	AoCoAoToTsGsCsTsGsGsGsCsTsTsCsAoGoGoToC	71	0521-0540
100809	AoToCoCoTsCsAsGsCsTsCsGsCsAsGsToCoCoToC	72	0551-0570
100810	ToAoCoCoAsCsCsGsTsGsTsGsGsCsCsAoCoAoToA	73	0617-0636
100811	CoAoGoToTsTsAsGsCsAsTsGsAsTsCsToCoToGoG	74	0644-0663

100812	CoAoGoGoCsCsAsCsAsGsAsCsCsAsGsAoToGoToC	75	0686-0705
100813	CoCoToToCsCsAsGsCsAsGsTsTsCsAsAoGoCoCoA	76	0711-0730
101123	CoAoGoCoAsCsCsAsTsGsGsAsCsGsCsGoGoAoAoC	77	21871 mismatch

[1] Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-), including 2'-MOE and 2'-deoxy residues, 5-methyl-cytosines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester.

[2] Co-ordinates from Genbank Accession No. AI119044, locus name "AI119044", SEQ ID NO. 61.

Mouse p38β antisense sequences were screened in bEND.3 cells as described in Example 4. Results are shown in Table 11.

Oligonucleotides 100800 (SEQ ID NO. 63), 100801 (SEQ ID NO. 64), 100803 (SEQ ID NO. 66), 100804 (SEQ ID NO. 67), 100805 (SEQ ID NO. 68), 100807 (SEQ ID NO. 70), 100808 (SEQ ID NO. 71), 100809 (SEQ ID NO. 72), 100810 (SEQ ID NO. 73), 100811 (SEQ ID NO.74), and 100813 (SEQ ID NO. 76) resulted in at least 50% inhibition of p38β mRNA expression. Oligonucleotides 100801 (SEQ ID NO.64), 100803 (SEQ ID NO. 66), 100804 (SEQ ID NO. 67), 100805 (SEQ ID NO. 68), 100809 (SEQ ID NO. 72), and 100810 (SEQ ID NO. 73) resulted in at least 70% inhibition and are preferred. Oligonucleotides 100801 (SEQ ID NO. 64), 100805 (SEQ ID NO. 68), and 100811 (SEQ ID NO. 74) resulted in significant inhibition of p38α mRNA expression in addition to their effects on p38β.

TABLE 11

Inhibition of Mouse p38 mRNA expression in bEND.3 Cells by Chimeric (deoxy gapped) Mixed Backbone p38β Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	% p38β mRNA INHIBITION	% p38α mRNA INHIBITION
control	---	0%	0%
100800	63	51%	---
100801	64	74%	31%
100802	6[illegible]	35%	---
100803	66	74%	18%
100804	67	85%	18%
100805	68	78%	58%
100806	69	22%	3%
100807	70	64%	---
100808	71	53%	13%

ISIS No:	SEQ ID NO:	% p38β mRNA INHIBITION	% p38α mRNA INHIBITION
100809	72	84%	14%
100810	73	72%	1%
100811	74	60%	43%
100812	75	36%	17%
100813	76	54%	---

Example 6: Effect of p38 MAPK Antisense Oligonucleotides on IL-6 Secretion

p38 MAPK antisense oligonucleotides were tested for their ability to reduce IL-6 secretion. bEND.3 cells were cultured and treated as described in Example 4 except that 48 hours after oligonucleotide treatment, cells were stimulated for 6 hours with 1 ng/mL recombinant mouse IL-1 (R&D Systems, Minneapolis, MN). IL-6 was measured in the medium using an IL-6 ELISA kit (Endogen Inc., Woburn, MA).

Results are shown in Table 13. Oligonucleotides targeting a specific p38 MAPK isoform were effective in reducing IL-6 secretion greater than approximately 50%.

Table 13: Effect of p38 Antisense Oligonucleotides on IL-6 secretion

ISIS No:	SEQ ID NO:	GENE TARGET	DOSE (μM)	%IL-6 INHIBITION
control	---	---		0%
21873	52	p38α	100	49%
100804	67	p38β	100	57%
21871	50	p38α and p38β	200	23%

EXAMPLE 7: Activity of p38α Antisense Oligonucleotides in Rat Cardiomyocytes

Rat p38α antisense oligonucleotides were screened in

Rat A-10 cells. A-10 cells (American Type Culture Collection, Manassas, VA) were grown in high-glucose DMEM (Life Technologies, Gaithersburg, MD) medium containing 10% fetal calf serum (FCS). Cells were treated with oligonucleotide as described in Example 2. Oligonucleotide concentration was 200 nM. mRNA was isolated 24 hours after time zero and quantitated by Northern blot as described in Example 2.

Results are shown in Table 14. Oligonucleotides 21845 (SEQ ID NO. 48), 21846 (SEQ ID NO. 49), 21871 (SEQ ID NO. 50), 21872 (SEQ ID NO. 51), 21873 (SEQ ID NO. 52), 21874 (SEQ ID NO. 53), 21875 (SEQ ID NO. 54), 21877 (SEQ ID NO. 56), 21878 (SEQ ID NO. 57), 21879 (SEQ ID NO. 58), and 21881 (SEQ ID NO. 60) inhibited p38α mRNA expression by 65% or greater in this assay. Oligonucleotides 21846 (SEQ ID NO. 49), 21871 (SEQ ID NO. 50), 21872 (SEQ ID NO. 51), 21877 (SEQ ID NO. 56), and 21879 (SEQ ID NO. 58) inhibited p38α mRNA expression by greater than 85% and are preferred.

TABLE 14

Inhibition of Rat p38α mRNA expression in A-10 Cells by Chimeric (deoxy gapped) Mixed Backbone p38α Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% p38α mRNA EXPRESSION	% p38α mRNA INHIBITION
control	---	---	100%	0%
21844	47	AUG	75%	25%
21845	48	coding	25%	75%
21846	49	coding	8%	92%
21871	50	coding	12%	88%
21872	51	coding	13%	87%
21873	52	stop	19%	81%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% p38α mRNA EXPRESSION	% p38α mRNA INHIBITION
21874	53	3'-UTR	22%	78%
21875	54	3'-UTR	26%	74%
21876	55	3'-UTR	61%	39%
21877	56	3'-UTR	12%	88%
21878	57	3'-UTR	35%	65%
21879	58	3'-UTR	11%	89%
21881	60	3'-UTR	31%	69%

The most active oligonucleotide in this screen (SEQ ID NO. 49) was used in rat cardiac myocytes prepared from neonatal rats (Zechner, D., *et. al., J. Cell Biol.*, **1997**, *139*, 115-127). Cells were grown as described in Zechner et al. and transfected with oligonucleotide as described in Example 2. Oligonucleotide concentration was 1 μM. mRNA was isolated 24 hrs after time zero and quantitated using Northern blotting as described in Example 2. An antisense oligonucleotide targeted to JNK-2 was used as a non-specific target control.

Results are shown in Table 15. Oligonucleotide 21846 (SEQ ID NO. 49) was able to reduce p38α expression in rat cardiac myocytes by nearly 60%. The JNK-2 antisense oligonucleotide had little effect on p38α expression.

TABLE 15

Inhibition of Rat p38α mRNA expression in Rat Cardiac Myocytes by A Chimeric (deoxy gapped) Mixed Backbone p38α Antisense Oligonucleotide

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% p38α mRNA EXPRESSION	% p38α mRNA INHIBITION
control	---	---	100%	0%
21846	49	coding	41%	59%

EXAMPLE 8: Additional Human p38α Oligonucleotide Sequences

Additional antisense oligonucleotides were designed to target human p38α based on active rat sequences. Target sequence data are from the p38 MAPK cDNA sequence; Genbank accession number L35253, provided herein as SEQ ID NO: 1. Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All 2'-MOE cytosines and 2'-OH cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 16.

TABLE 16:

Additional Nucleotide Sequences of Human p38α Chimeric (deoxy gapped) Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
100860	**CTGAG**ACATTTTCCA**GCGGC**	78	0284-0303	AUG
100861	**ACGCT**CGGGCACCTC**CCAGA**	79	0344-0363	coding
100862	**AGCTT**CTTCACTGCC**ACACG**	80	0439-0458	coding
100863	**AATGA**TGGACTGAAA**TGGTC**	81	0464-0483	coding
100864	**TCCAA**CAGACCAATC**ACATT**	82	0538-0557	coding
100865	**TGTAA**GCTTCTGACA**TTTCA**	83	0644-0663	coding
100866	**TGAAT**GTATATACTT**TAGAC**	84	0704-0723	coding
100867	**CTCAC**AGTCTTCATT**CACAG**	85	0764-0783	coding
100868	**CACGT**AGCCTGTCAT**TTCAT**	86	0824-0843	coding
100869	**CATCC**CACTGACCAA**ATATC**	87	0907-0926	coding
100870	**TATGG**TCTGTACCAG**GAAAC**	88	0960-0979	coding
100871	**AGTCA**AAGACTGAAT**ATAGT**	89	1064-1083	coding
100872	**TTCTC**TTATCTGAGT**CCAAT**	90	1164-1183	coding
100873	**CATCA**TCAGGATCGT**GGTAC**	91	1224-1243	coding
100874	**TCAAA**GGACTGATCA**TAAGG**	92	1258-1277	coding
100875	**GGCAC**AAAGCTGATG**ACTTC**	93	1324-1343	coding
100876	**AGGTG**CTCAGGACTC**CATCT**	94	1364-1383	stop
100877	**GCAAC**AAGAGGCACT**TGAAT**	95	1452-1471	3'-UTR

[1] Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-) including "C" and "C" residues, 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Co-ordinates from Genbank Accession No. L35253, locus name "HUMMAPKNS", SEQ ID NO. 1.

The compounds shown in Table 16 were screened by Northern blot analysis in human T24 urinary bladder cancer cells for ability to inhibit human p38α expression. Cells were obtained from the American Type Culture Collection (Manassas VA). Oligonucleotide concentration was 200 nM, transfection was in the presence of cationic lipid and RNA was isolated 24 hours after oligonucleotide treatment. Oligonucleotides were also tested for ability to inhibit human p38β expression in T24 cells as a measure of specificity for the p38α target. Results are shown in Table 17.

Table 17

Inhibition of Human p38α mRNA Expression in T24 Cells by Chimeric p38α Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	% p38α EXPRESSION	% p38β EXPRESSION	% p38α INHIB.	% p38β INHIB.
100860	78	73	71	27	29
100861	79	60	47	40	53
100862	80	56	45	44	55
100863	81	49	67	51	33
100864	82	66	70	34	30
100865	83	64	63	36	37
100866	84	55	65	45	35
100867	85	58	33	42	67
100868	86	47	60	53	40
100869	87	61	100	39	--
100870	88	51	n.d.	49	n.d.
100871	89	57	96	43	4
100872	90	37	77	63	23
100873	91	34	70	66	30
100874	92	42	76	58	24
100875	93	39	90	61	10

ISIS No:	SEQ ID NO:	% p38α EXPRESSION	% p38β EXPRESSION	% p38α INHIB.	% p38β INHIB.
100876	94	77	93	23	7
100877	95	47	95	53	5

ISIS 100863, 100868, 100872, 100873, 100874, 100875 and 100877 all inhibited the p38α target by over 50%.

Example 9: Dose responses for Human p38α antisense oligonucleotides

Dose response experiments were performed for ISIS 100872, 100873, 100874 and 100875. Results are shown in Table 18.

Table 18

Dose responses of human p38α oligonucleotides

ISIS #	Oligo conc(nM)	% Inhib of p38α	% Inhib of p38β	SEQ ID NO:
100872	200	83	8	90
	100	82	9	90
	50	55	--	90
100873	200	92	56	91
	100	88	22	91
	50	81	10	91
100874	200	87	49	92
	100	73	--	92
	50	53	--	92
100875	200	92	31	93
	100	87	8	93
	50	70	--	93

Example 10: Additional Human p38β Oligonucleotide Sequences

Additional antisense oligonucleotides were designed to target human p38β (target sequence is Genbank accession number U53442, provided herein as SEQ ID NO: 23).

Oligonucleotides was synthesized as chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the deoxy gap region and phosphodiesters in the wings. All 2'-MOE cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 19.

Table 19

Additional Human p38β Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
10786	ACAGACGGAGCCGTAGGCGC	96	117-136	Coding
10787	CACCGCCACCTTCTGGCGCA	97	156-175	Coding
10787	GTACGTTCTGCGCGCGTGGA	98	207-226	Coding
10787	ATGGACGTGGCCGGCGTGAA	99	287-306	Coding
10787	CAGGAATTGAACGTGCTCGT	100	414-433	Coding
10787	ACGTTGCTGGGCTTCAGGTC	101	491-510	Coding
10787	TACCAGCGCGTGGCCACATA	102	587-606	Coding
10787	CAGTTGAGCATGATCTCAGG	103	614-633	Coding
10787	CGGACCAGATATCCACTGTT	104	649-668	Coding
10787	TGCCCTGGAGCAGCTCAGCC	105	682-701	Coding

The compounds shown in Table 19 were screened by Northern blot analysis in human T24 urinary bladder cancer cells for ability to inhibit human p38β expression. Cells were obtained from the American Type Culture Collection (Manassas VA). Oligonucleotide concentration was 200 nM, transfection was in the presence of cationic lipid and RNA was isolated 24 hours after oligonucleotide treatment.

Oligonucleotides were also tested for ability to inhibit human p38α expression in T24 cells as a measure of specificity for the p38β target. Results are shown in Table 20

Table 20

Inhibition of Human p38β mRNA Expression in T24 Cells by Chimeric p38β Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	% p38β EXPRESSION	% p38α EXPRESSION	% p38β INHIB.	% p38α INHIB.
107869	96	60	93	40	7
107870	97	74	97	26	3
107871	98	60	111	39	--
107872	99	57	123	43	--
107873	100	58	120	42	--
107874	101	61	100	39	0
107875	102	92	112	8	--
107876	103	127	137	--	--
107877	104	n.d.	n.d.	--	--
107878	105	54	112	46	--

"n.d." indicates no data.

ISIS 107869, 107872, 107873, 107874 and 107878 inhibited p38β expression by at least about 405 in this experiment. However, because of technical difficulties with this experiment, ISIS 107871, 107872, 107873, 107874, 107875, 107877 and 107878 were retested in dose response assays. This is described in Example 11.

Example 11: Dose responses for Human p38β antisense oligonucleotides

Dose response experiments were performed for ISIS 107871, 107872, 107873, 107874, 107875, 107877 and 107878. Results are shown in Table 21.

Table 21

Dose responses of human p38β oligonucleotides

ISIS #	Oligo conc(nM)	% Inhib of p38β	% Inhib of p38α	SEQ ID NO:
107871	200	90	--	98
	100	58	--	98
	50	59	--	98
107872	200	78	--	98
	100	87	16	98
	50	29	--	99
107873	200	77	--	100
	100	59	--	100
	50	31	--	100
107874	200	77	13	101
	100	66	1	101
	50	25	--	101
107875	200	60	9	102
	100	62	--	102
	50	18	7	102
107877	200	73	--	104
	100	66	--	104
	50	50	--	104
107878	200	73	--	105
	100	57	--	105
	50	30	--	105

Example 12

Specificity of antisense inhibitors of p38α and p38β for the isoform to which they are targeted

Additional dose response curves were done for ISIS 100872 (targeted to p38α) and ISIS 107871 (targeted to p38β) inhibition of human both p38α and p38β expression in

T24 cells. Data are shown in Table 22 and confirm that both compounds have high activity and specificity against their isoform target.

Table 22:

Dose response curves for ISIS 100872 and ISIS 107871 inhibition of p38α and p38β expression

ISIS #	Oligo conc(nM)	% Inhib of p38α	% Inhib of p38β	SEQ ID NO:
100872	0	0	0	90
	50	54	--	90
	100	82	8	90
	200	83	8	90
107871	0	0	0	98
	50	--	29	98
	100	16	86	98
	200	0	78	98

What is claimed is:

1. An antisense compound 8 to 30 nucleobases in length targeted to the 5'-untranslated region, translational start site, translational termination region or 3'-untranslated region of a nucleic acid molecule encoding a p38 mitogen activated protein kinase, wherein said antisense compound inhibits the expression of said p38 mitogen activated protein kinase.

2. The antisense compound of claim 1 which is an antisense oligonucleotide.

3. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least an 8-nucleobase portion of SEQ ID NO: 17, 20, 38, 39, 41, 42, 43 or 95.

4. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

5. The antisense compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

6. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

7. The antisense compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl moiety.

8. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

9. The antisense compound of claim 8 wherein modified nucleobase is a 5-methyl cytosine.

10. The antisense compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

11. A pharmaceutical composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

12. The pharmaceutical composition of claim 11 further comprising a colloidal dispersion system.

13. The pharmaceutical composition of claim 11 wherein the antisense compound is an antisense oligonucleotide.

14. A method of inhibiting the expression of a p38 mitogen activated protein kinase in cells or tissues comprising contacting said cells or tissue with the antisense compound of claim 1 so that expression of said p38 mitogen activated protein kinase is inhibited.

15. A method of treating an animal having a disease or condition associated with a p38 mitogen activated protein kinase comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of said p38 mitogen-activated protein kinase is inhibited.

16. The method of claim 15 wherein the disease or condition is an inflammatory or autoimmune disease.

17. The method of claim 16 wherein said inflammatory or autoimmune disease or condition is rheumatoid arthritis.

18. The method of claim 15 wherein said disease or condition is heart disease.

19. An antisense compound 8 to 30 nucleobases in length targeted to the coding region of a nucleic acid molecule encoding a p38 mitogen-activated protein kinase, wherein said antisense compound inhibits the expression of said p38 mitogen-activated protein kinase and comprises at least an 8-nucleobase portion of SEQ ID NO. 13, 26, 27, 28, 33, 35, 37, 81, 86, 90, 91, 92, 93, 96, 98, 99, 100, 101, 102, 104 or 105.

20. The antisense compound of claim 19 which is an antisense oligonucleotide.

21. The antisense compound of claim 20 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

22. The antisense compound of claim 21 wherein the modified internucleoside linkage is a phosphorothioate linkage.

23. The antisense compound of claim 20 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

24. The antisense compound of claim 23 wherein the modified sugar moiety is a 2'-O-methoxyethyl moiety.

25. The antisense compound of claim 20 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

26. The antisense compound of claim 25 wherein modified nucleobase is a 5-methyl cytosine.

27. The antisense compound of claim 20 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

28. A pharmaceutical composition comprising the antisense compound of claim 19 and a pharmaceutically acceptable carrier or diluent.

29. The pharmaceutical composition of claim 28 further comprising a colloidal dispersion system.

30. The pharmaceutical composition of claim 28 wherein the antisense compound is an antisense oligonucleotide.

31. A method of inhibiting the expression of p38 mitogen-activated protein kinase in cells or tissues comprising contacting said cells or tissue with the antisense compound of claim 19 so that expression of p38 mitogen-activated protein kinase is inhibited.

32. A method of treating an animal having a disease or condition associated with a p38 mitogen activated protein kinase comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of said p38 mitogen activated protein kinase is inhibited.

33. The method of claim 32 wherein the disease or condition is an inflammatory or autoimmune disease.

34. The method of claim 33 wherein said inflammatory or autoimmune disease or condition is rheumatoid arthritis.

35. The method of claim 32 wherein said disease or condition is heart disease.

36. An antisense compound 8 to 30 nucleobases in length targeted to p38α mitogen activated protein kinase, wherein said antisense compound inhibits the expression of said p38α mitogen activated protein kinase and does not substantially inhibit the expression of p38β mitogen activated protein kinase.

37. An antisense compound 8 to 30 nucleobases in length targeted to p38β mitogen activated protein kinase, wherein said antisense compound inhibits the expression of said p38β mitogen activated protein kinase and does not substantially inhibit the expression of p38α mitogen activated protein kinase.

38. The antisense compound of one of claims 1, 19, 36 or 37 wherein the p38 mitogen activated protein kinase is a human p38 mitogen activated protein kinase.

39. The antisense compound of one of claims 1, 19, 36 or 37 wherein the p38 mitogen activated protein kinase is a nonhuman animal p38 mitogen activated protein kinase.

40. The antisense compound of claim 39 wherein the nonhuman animal p38 mitogen activated protein kinase is a rodent p38 mitogen activated protein kinase.

SEQUENCE LISTING

<110> Monia, Brett P.
Gaarde, William A.
Nero, Pamela S.
McKay, Robert
Popoff, Ian
ISIS PHARMACEUTICALS, INC.

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Activated Protein Kinase Expression

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/08794

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04, 21/02; C12Q 1/68; C12N 5/00; A01N 43/04; A61K 31/70
US CL : 536/23, 24.31, 24.33, 24.5; 435/6, 91, 31, 325, 375; 514/55
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.1, 24.31, 24.33, 24.5; 435/6, 91, 31, 325, 375; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	COHEN et al. The Critical Role of -38 MAP Kinase in T Cell HIV-1 Replication. Molecular Medicine. May 1997, Vol. 3, Nol. 5, pages 339-346, especially page 342.	19-22 and 31 — 23-27
A	CROOKE, S.T. Basic Principles of Antisense Therapeutics. In: Antisense Research and Applications, Springer-Verlag Press, Berlin, Heidelberg, & New York. July 1998, pages 1-49, especially page 3.	1-40
A	BRANCH, A. A good antisence molecule is hard to find. TIBS. February 1998, Vol. 23, pages 45-50, especially page 45.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	07 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer George Elliott George Bridgers for Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/08794

Continuation of B. FIELDS SEARCHED Item3: USPAT, EPO, JPO, DERWENT, DIALOG, CAplus
Search terms: mitogen activated protein kinase, p38, inhibit, antiagonist, antisense, aptamer, triplex, AS-OSN, oligonucleotide, ribozyme